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Characterization of Human Immunodeficiency Virus Type 1 *vif* Gene in Long-Term Asymptomatic Individuals

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We have determined the sequence of the human immunodeficiency virus type 1 (HIV-1) *vif* genes from a cohort of 42 long-term nonprogressors (LTNP) and compared these sequences to those of 8 late progressors. The coding potential of the *vif* open reading frame directly derived by nested PCR from uncultured peripheral blood mononuclear cell DNA was conserved in all 50 individuals. The nucleotide distances between *vif* sequences were not significantly different between LTNP and late progressors, indicating similar selections of viruses within both types of long-term HIV-1-infected subjects. However, a statistically significant correlation between an amino acid signature at position 132 of Vif and the viral load was found within LTNP. Namely, amino acid Ser was associated with low viral load and amino acid Arg with high viral load. This signature was also observed when LTNP with low viral load were compared to progressors. The Ser132 signature was introduced in place of Arg132 present in the HIV-1 YU-2 Vif prototype into chimeric viruses to assess the impact of Vif signature on the virus. While the replication properties in the SupT1 cell line were unmodified, the mutagenized virus revealed a fivefold decreased replication in activated PBMC, suggesting a possible role of this Vif signature for viral production *in vivo*.

INTRODUCTION

Five to ten percent of human immunodeficiency virus type 1 (HIV-1)-infected individuals remain asymptomatic without anti-retrovirus therapy for more than 10 years and present stable levels of peripheral blood CD4-positive cells greater than 500 per mm^3. The long-term nonprogressors (LTNP) identified in several studies (Buchbinder *et al.,* 1994; Cao *et al.,* 1995; Learmont *et al.,* 1992; Lifson *et al.,* 1991; Sheppard *et al.,* 1993) can constitute a distinct group which may not develop AIDS or can correspond to the slowest progressors among the normal distribution of HIV-1-infected individuals.

Initial studies on viruses isolated from LTNP have revealed defects in *nef* (Deacon *et al.,* 1995; Kirchhoff *et al.,* 1995; Mariani *et al.,* 1996; Premkumar *et al.,* 1996), in *rev* (Iversen *et al.,* 1995), in *vpr, vif,* and *vpu* (Michael *et al.,* 1995a; Wang *et al.,* 1996), and in *env* (Connor *et al.,* 1996) genes, suggesting that those individuals may be infected by attenuated viruses. More recent compilations of nucleotide sequences of *gag-pol, vif, vpr, vpu, env, nef,* and LTR of HIV-1 from 10 LTNP failed to confirm the presence of defective genes and revealed overall intact HIV-1 genes within these individuals (Connor *et al.,* 1996; Huang *et al.,* 1995a,b, 1998; Zhang *et al.,* 1997a,b). Moreover, they did not find any clearcut correlation between the genotypes and the disease status, which was confirmed in the extensive study on the structure and function of *nef* within another cohort by Michael *et al.* (1995b). However, all of these studies have analyzed a limited number of individuals and have not assessed the possible relation between viral nucleotide sequences and the plasma viral load.

We report here our results on *vif* gene sequences from 42 LTNP untreated with anti-retroviral therapy (referred to here as the ALT cohort; (Candotti *et al.,* 1999). This auxiliary gene is required for HIV-1 replication in primary lymphocytes and macrophages (Chowdhury *et al.,* 1996; Fisher *et al.,* 1987; Gabuzda *et al.,* 1992, 1994; Sakai *et al.,* 1988; Sova and Volsky, 1993; von Schwedler *et al.,* 1993) and consequently is largely maintained intact *in vivo* in adults (Sova *et al.,* 1995; Wieland *et al.,* 1994; Zhang *et al.,* 1997a) and in perinatally infected infants (Yedavalli *et al.,* 1998). Our goal was to determine whether defined amino acid residues at particular positions of the predicted *vif* gene product were related to the viral load parameter among LTNP individuals. We found a statistically significant correlation between one precise amino acid signature at position 132 of Vif and the viral load within the LTNP. This signature was also observed when LTNP with

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TABLE 1

Clinical Characteristics of LVL and HVL LTNP and of the Eight Late Progressors

Viral load and CD4 counts	LVL LTNP (n = 18)	HVL LTNP (n = 24)	Late progressors (n = 8)
Plasma viral load mean (RNA copies/ml)	2273	189,858	305,850
Range	20–7800	11,000–860,000	2200–1,860,000
Blood CD4 cell number (cells/μl)	728	637	265
Range	434–1210	278–1153	158–354

Note. Plasma viral loads (RNA copies per milliliter) and absolute CD4 T cell counts (cells per microliter) were measured within 1 year after enrollment, as described by Candotti *et al.* (1999). No anti-retroviral therapy was prescribed before this first cross sectional survey. Mean and range of values were calculated for the three groups of individuals.

low viral load were compared to progressors. When Ser132 in place of Arg132 present in HIV-1 YU-2 Vif prototype was introduced into a chimeric virus to assess the impact of the Vif signature on the replication properties of HIV-1 in activated peripheral blood mononuclear cells (PBMC), the mutagenized virus revealed a fivefold reduced replication, suggesting a possible role of this Vif signature for virus production *in vivo.*

RESULTS

Definition of the asymptomatic subjects

The group of LTNP in this study consisted of 42 subjects HIV-1 infected since at least 8 years with stable CD4 cell counts (no significant decline of this count in the past 3 years). In parallel, 8 HIV-1-positive subjects with similar duration of HIV-1 infection but exhibiting significant declines of CD4 cell count in the past 3 years (decline of at least 25% during this period) were used as late progressor controls. Viral loads and CD4 cell counts of all subjects were determined within the first year after inclusion in the cohort. According to the viral load parameters (Table 1), the 42 LTNP were divided into two groups, 18 low viral load (LVL) LTNP and 24 high viral load (HVL) LTNP. LVL LTNP individuals presented viral loads inferior or equal to 10,000 copies/ml and HVL LTNP shared viral loads greater than 10,000 copies/ml. Viral load means corresponded to 2273, 189,858, and 305,850 RNA copies/ml within the three respective groups, LVL LTNP, HVL LTNP, and late progressors. CD4 T-cell count means were similar in both LVL and HVL groups (728 and 637). The late progressor group presented a lower CD4 T-cell count mean equal to 265.

vif sequence data obtained and deduced Vif proteins

Proviral DNA as well as viral RNA was purified, respectively, from PBMC and from plasma viral particles. Nested *Pfu* PCR or RT-PCR allowed us to obtain a DNA fragment corresponding to the *vif* open reading frame (576 nt). The DNA fragments were directly sequenced. The majority of *vif* sequences were obtained from PBMC proviral DNA (denominated A1) and a few from plasma viral RNA (denominated B1). All sequences except one were taken from individuals within the first year after inclusion into the cohort. One single HVL LTNP PBMC sample vi04048A2H was taken within the second year after inclusion. Three LVL *vif* sequences, vi06002C1L, vi04005C1L, and vi11007C1L, were derived from PBMC cocultures, the only way to successfully amplify the proviral sequences. Figure 1 shows the alignment of Vif amino acid sequences deduced from the nucleotide sequences. In addition to the Vif sequences from the eight control progressors of our cohort, seven Vif sequences, which were obtained by amplification of PBMC DNA from AIDS patients and were published by Sova *et al.* (1995), were also aligned (Vif GMK1 to 4, VifGMK6 to 8). The major amino acid sequence of all subjects was found to be full-length, i.e., 192 amino acids long except for one subject (11007) who presents a Vif protein with 193 amino acids with an additional D at position 62.

As observed previously by various authors, different discrete regions, amino acids 27–52, 60–67, 92–102, 120–137, and 151–167, were particularly variable. Between these regions, conserved motifs were observed, in particular the SLQXLA motif located between amino acids 144 and 149 (Oberste and Gonda, 1992) and two other motifs in the last 30 C-terminal amino acids of the Vif protein, i.e., ^{161}PPLPS^{165} and ^{168}KLTEDRWN^{175}. These two latter motifs are located in the domain which is in association with the plasma membrane and with the Gag-precursor (Bouyac *et al.,* 1997; Goncalves *et al.,* 1995). Moreover Arg and Lys residues within the C-terminal end of Vif (8 to 11 residues between the positions 157 and 192), which are the most important residues for those interactions, are largely conserved in all LTNP as well as in late progressor Vif sequences. All LTNP sequences contain Ser^{144} and Thr^{188}, which are the sites of serine threonine protein kinases (Yang *et al.,* 1996), as well as Thr^{96} and Ser^{165}, which are the sites of the MAP kinase (Yang and Gabuzda, 1998). Last, both cysteine residues which have been shown to be critical for the Vif function (Ma *et al.,* 1994) were conserved in all Vif proteins.

Phylogenetic relationship of *vif* sequences and genetic diversity

Figure 2 shows the dendogram, drawn by the neighbor joining method, of all *vif* sequences, with the YU-2 *vif* sequence as outgroup. Nucleotide sequences from the LTNP subjects interdigitated with those of progressors and AIDS patients without apparent strong grouping among themselves. Moreover, the LTNP subjects were analyzed in relation to their virus load and no clear

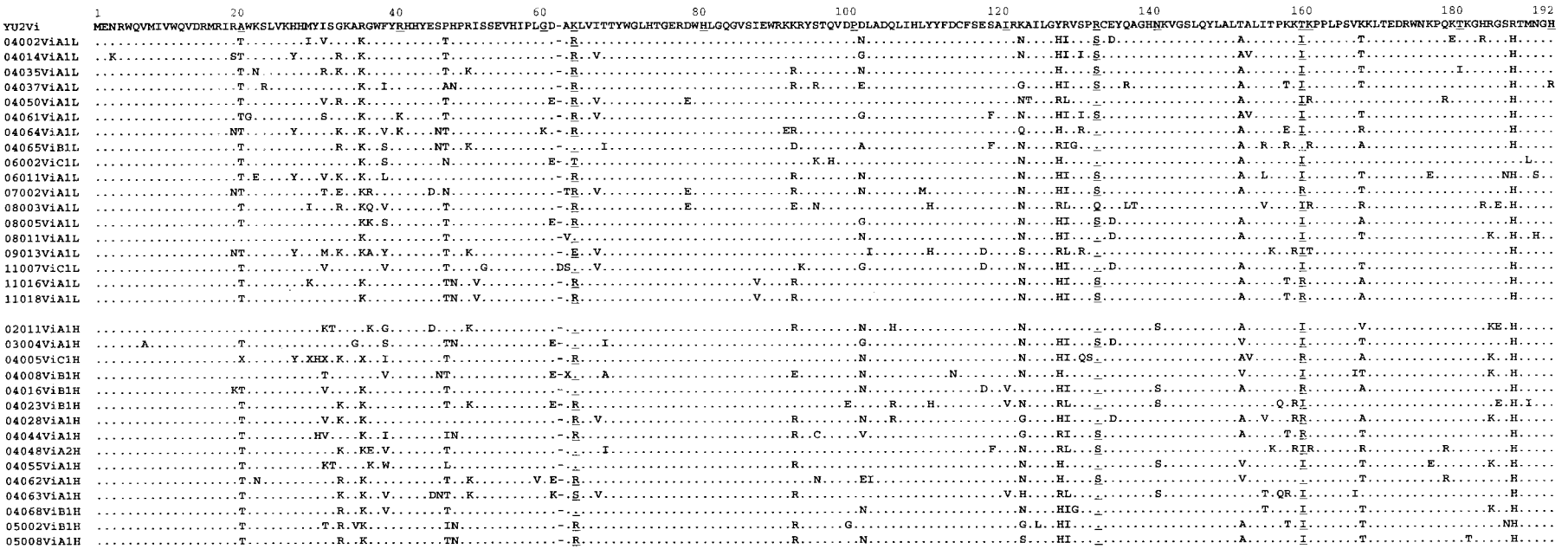

FIG. 1. Multiple alignment of deduced amino acid sequences for the *vif* gene of HIV-1-infected subjects. Sequences were grouped as LVL (L) and HVL (H) LTNP and as late progressors (T). Numbers denote individual sequences while added letters A, B, and C, denote the source of the viral sequence: A, DNA from PBMC; B, RNA from plasma; and C, DNA from cultured PBMC. An additional number 1 or 2 denotes the time of analysis after enrollment in the ALT cohort: 1, for 1 year; 2, for 2 years (Candotti *et al.,* 1999). Sequences of AIDS progressors from the study of Sova *et al.* (1995), which were obtained by PCR amplification from *ex vivo* PBMC, were added as reference sequences of progressors. Dots indicate amino acids identical to those of the YU-2 sequence (Li *et al.,* 1992); dashes represent gaps. Rare unidentified amino acids are indicated with an X. Most variable amino acid positions in our study (see Table 2), i.e., positions 63, 132, and 159, were underlined in each Vif-deduced sequence. Below multiple alignments, phosphorylated residues previously described (Yang and Gabuzda, 1998; Yang *et al.,* 1996) and SLQYL and PPLP (Oberste and Gonda, 1992) motifs are reiterated.

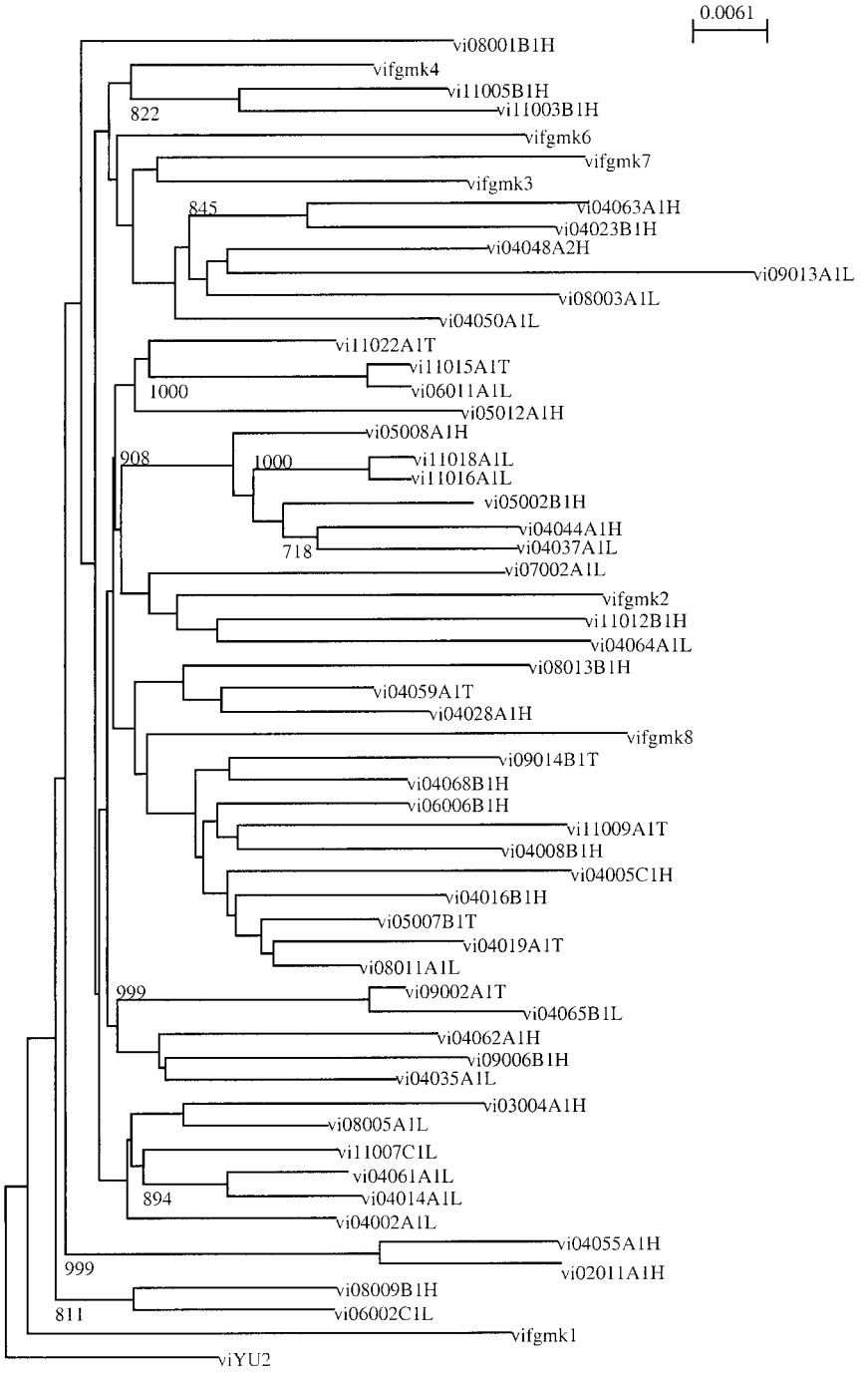

FIG. 2. Phylogenetic tree of *vif* sequences from LTNP and late progressors. The tree of nucleotide sequences was constructed using ClustalW software. The sequences are labeled as in Fig. 1. The outgroup sequence was the reference HIV-1 YU-2 sequence (Li *et al.,* 1992). Significant boostrapping values are indicated.

subclusters were revealed within LVL and HVL LTNP. Of note, sequence analysis of the envelope C2V3 domain revealed that all subjects who were currently analyzed (i.e., 34 LTNP + 6 progressors) were infected by B subtype viruses (D. Candotti, personal communication) and, consequently, the variability of *vif* gene could not be related, in our study, to differences in HIV-1 clades.

Comparative analysis of amino acid signature patterns between LVL and HVL LTNP and between LTNP and progressors

To investigate whether specific amino acids in Vif are associated with different statuses of infected individuals (LVL, HVL LTNPs, and progressors), the amino acid signature pattern in the Vif amino acid sequences was

TABLE 2

Signature Pattern Analysis of LVL and HVL LTNP and Progressors

	Number of samples with the indicated residues at six critical positions of Vif amino acid sequence according to the group of subjects												
	33		63		92		101		132		159		
Subject group comparison[b]	R	G	R	K	K	R	D	N	S	R	I	T	Number of subjects
LVL LTNP	4	9			9	7			■ 10	■ 7			18
HVL LTNP	9	8			8	11			6	18			24
LTNP			■ 27	12			17	14			■ 27	■ 6	42
Progressors			5	7			4	6			5	6	15
LVL LTNP			■ 13	3	9	7	7	5	■ 10	■ 7	■ 14	■ 1	18
Progressors			5	7	4	7	4	6	3	12	5	6	15
HVL LTNP	9	8	14	9			14	9			7	5	24
Progressors	4	8	5	7			4	6			5	6	15
Zhang LTNP									■ 6	■ 2	■ 7	1	9
Progessors									3	12	5	6	15
LVL LTNP + Zhang LTNP			17	8	13	12			■ 16	■ 9	■ 21	■ 2	27
Progessors			5	7	4	7			3	12	5	6	15
All LTNP			□ 31	17							■ 34	■ 7	51
Progessors			5	7							5	6	15

[a] The amino acid signatures relative to different subject group comparisons were determined by VESPA software (Korber and Myers, 1992) and tested for their statistical significance by χ^2 test using Statview (Abacus Concepts). Closed squares indicate pairs with probability values less than 0.05 in the χ^2 test. Open squares indicate comparison pairs, which give probability values comprised between 0.05 and 0.08. When χ^2 test gives *P* values less than 0.05, for both compared residues versus other residues at one position (for example, S and R at position 132), the position is considered a significant signature for a subject group.

[b] Zhang LTNP correspond to nine LTNP described by Zhang *et al.* (1997b). Progressors correspond to the eight late progressors of the present study and to seven AIDS progressors from Sova *et al.* (1995) (see Fig. 1).

carefully analyzed within the different groups of individuals (Table 2). The consensus Vif sequences from nine long-term survivors (LTNP), described in the study of Zhang *et al.* (1997b) and corresponding to LTNP with low viral load, were also grouped and compared to sequences of progressors. The sequence comparisons were performed with the VESPA algorithm (Korber and Myers, 1992) and tested for their statistical significance by the χ^2 test.

Six amino acid positions were revealed when Vif sequences were compared in different ways as shown in Table 2. The most statistically significant signatures were restricted to positions 132 and 159. More precisely, amino acid S at position 132 was correlated with the LVL LTNP status, and alternative R residue was associated with the HVL LTNP and progressor status. The I residue of the position 159 was predominant within the LVL LTNP, the LTNP of Zhang's study, and within overall LTNP sequences while the T residue marked the progressor sequences. However, the residue at position 159 did not distinguish the LVL from HVL sequences. In conclusion, amino acid S at position 132 was associated with LTNP of low viral load and R at the same position was associated with LTNP of high viral load and with progressor individuals. In contrast, amino acids I and T at position 159 were associated with nonprogressors and progressors, respectively, independently of the viral load parameter.

Correlation between viral load in LTNP individuals and amino acid signature at position 132

The statistical significance of the amino acid signature at position 132 was further assessed by estimating a possible correlation between measured viral loads of each LTNP individual (not shown in Table 1) and the signatures of position 132 by Mann–Whitney and Kruskall–Wallis testing. We confirmed that the signatures S and R at position 132 are highly associated with the viral load parameter within LTNP individuals (S with low viral load, R with high load), while the other signatures, including I and T of position 159, are not (Table 3). The mean viral load for the 16 LTNP individuals containing S at position 132 was 38,096 ±

TABLE 3

Correlation between Viral Loads within LTNP Individuals and Amino Acid Signatures at Position 132

Vif signature within individuals	LTNP	Number of individuals	Mean viral load ± standard error[a]
Position 33	G	17	113,376 ± 47,907
	R	13	61,397 ± 27,168
	K	11	169,995 ± 76,102
	E	1	2000
Position 63	R	27	83,258 ± 28,147
	K	12	123,461 ± 47,354
	T	1	210
	E	1	7800
	S	1	860,000
Position 92	R	18	93,070 ± 47,306
	K	17	96,032 ± 37,645
	E	6	214,933 ± 100,396
	D		115
Position 101	D	17	148,286 ± 59,397
	N	14	86,032 ± 42,028
	G	6	43,363 ± 35,642
	E	2	170,045 ± 169,955
	A	1	115
	V	1	110,000
	S	1	162,000
Position 132	S	16	38,096 ± 1536
	R	25	159,287 ± 44,590
	Q	1	5,800
Position 159	I	27	110,577 ± 40,410
	T	6	89,952 ± 54,162
	R	9	119,135 ± 55,410

[a] Statistical data were obtained with Mann–Whitney and Kruskall–Wallis tests. Square symbols indicate the S/R pair comparison which was statistically significant using the Mann–Whitney test ($P < 0.05$).

21,536, while the mean viral load for the 25 individuals containing R was 159,287 ± 44,590 with a good statistical significance using the Mann–Whitney test ($P = 0.018$) (when the single individual with residue Q at position 132 was excluded) and the Kruskall–Wallis test ($P = 0.05$) when the individual with Q was maintained. In contrast, as expected, no correlation was measured for all other positions.

Moreover, any important bias regarding the nature of the source material for amplification, i.e., PBMC DNA or plasma RNA, has been excluded. Indeed, a comparative analysis of S and R signatures in the Vif proteins deduced from both source materials of 10 individuals revealed that 8 patients had unchanged signatures (6 with R and 2 with S) while only 2 patients had distinct signatures with an S in PBMC DNA and an R in plasma RNA (data not shown).

We could therefore conclude that the signature at position 132 is significantly correlated to the viral load in a LTNP environment and that the S signature may confer an attenuation of replication rather than a complete defectiveness in PBMC.

Functional assay of the amino acid signatures S and R of position 132 of the YU-2 Vif protein

To address the role of this signature in the replication properties of HIV-1, chimeric viruses containing the residues S or R at position 132 of an appropriate *vif* gene product were constructed. The parental provirus corresponded to a *vif* minus genome of HIV-1 NDK strain and the inserted *vif* gene of subtype B HIV-1 was that of YU2 HIV-1, where R at position 132 was mutated into S. After transfection of 293 cells, the reconstructed viruses were grown in SupT1 cells permissive for both *vif* minus and plus viruses. The replication properties of both reconstructed viruses in these permissive cells were identical depending only on infectious dose and not on cloned *vif* gene (data not shown). The virus stocks were titrated on C8166 cells and viruses were then assayed in PBMC restrictive for *vif* minus viruses at two multiplicities of infection (m.o.i.) of 0.05 and 0.01 TCID/cell corresponding, respectively, to 7.5 and 1.5×10^4 TCID/1.5×10^6 cells. Virus production was monitored twice a week by measuring RT activity in infected cell culture supernatants (Fig. 3). Production of virus expressing 132S-mutated YU-2 Vif protein was reproducibly lower than that of corresponding virus expressing R132 YU-2 Vif protein in triplicate parallel infected cell cultures repeated three times with a mean ratio of 5.1 ± 0.5 in restrictive PBMC at day 8 postinfection. The average fivefold decrease in viral production was correlated to a decrease in infectivity by measuring on C8166 the infectivity present in the supernatants of PBMC at day 8 postinfection, after normalization for the reverse transcriptase activity. The mean ratio of normalized infectivities between R132 and S132 viruses grown in PBMC was 12.5 ± 2.5 based on triplicate parallel assays and on three different experiments. Using a similar approach we constructed NDK proviruses with nonmutated or mutated *vif* genes at position 159 from YU-2 with the mutation T159I. In replication assays of reconstructed viruses in PBMC, we could not observe any attenuating effect of the mutation T159I (data not shown). These different data allowed us to conclude that serine at position 132 of Vif plays a critical role in the decreased replication of Vif S132-containing viruses in restrictive PBMC.

DISCUSSION

A cross sectional analysis of HIV-1 *vif* sequences directly derived from the DNA of uncultured PBMC of 42 LTNP and 8 late progressors revealed a very high frequency of conserved intact open reading frames of the *vif* gene. By this approach, we analyzed the sequences of *vif* gene present *in vivo* without possible adaptation on *in vitro* cultivation. The two cysteines at position 114 and 133 (Ma *et al.,* 1994), the phosphorylation sites on Ser^{144} and Thr^{188} by serine-threonine kinases (Yang *et al.,* 1996) and on Thr^{96} and Ser^{165} by MAP kinase (Yang and

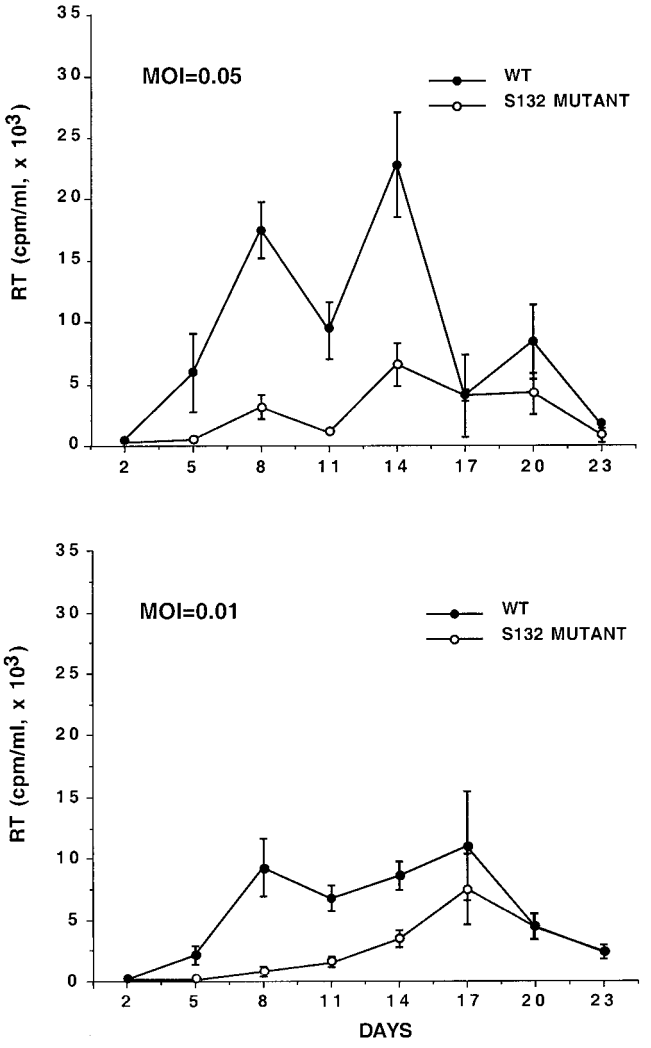

FIG. 3. Replication kinetics of chimera HIV-1 derived from full-length molecular clone HIV-1 NDK containing the *vif* gene of YU-2, wild type or R to S mutated at position 132, in PHA/IL2-stimulated PBMC. Viruses were prepared by transfecting HeLa cells with appropriate plasmids and by passaging (1×) virus contained in collected supernatants from transfected HeLa cells in permissive SupT1 cells. Resulting virus stocks were titrated in C8166 cells. PBMC cells (1.5×10^6) were infected in triplicate with 1 ml containing 7.5×10^4 and 1.5×10^4 TCID in parallel cultures (m.o.i. of 0.05 and 0.01). Virus replication was followed by RT activity measurement in cell-free supernatant twice weekly for 24 days. Data are expressed as mean values with standard errors of RT activities along triplicate parallel infections. Closed and open circles represent, respectively, nonmutated and mutated (R132S)YU-2 *vif* gene recombinant NDK viruses. Data represent means ± standard errors of three parallel cultures determined in one experiment.

Gabuzda, 1998), the series of Arg and Lys residues, and a motif ^{161}PPLP^{164} located in the C-terminus are well conserved in all isolates, confirming their importance in the function of Vif *in vivo*. Our results are consistent with the earlier published analysis of *vif* sequences from overall HIV-1-infected individuals (Sova *et al.,* 1995; Wieland *et al.,* 1994) as well as from LTNP (Zhang *et al.,* 1997b).

A significant correlation between plasma viral load within LTNP individuals and one particular amino acid signature in Vif sequences was determined in the present study. This finding was possible because for the first time a significant number of LTNP has been studied in the same cross sectional study. At position 132 of Vif, Ser is related to low viral load, while Arg is more related to high viral load. This signature can also distinguish LTNP with low viral load from progressors. That this signature was related at once to the viral load and to the progression status is not surprising since it is well known that the plasma viral load within infected individuals is significantly associated with progression rate toward AIDS (Connor *et al.,* 1993; Coombs *et al.,* 1989; Hogervorst *et al.,* 1995; Mellors *et al.,* 1997; O'Brien *et al.,* 1996; Piatak *et al.,* 1993; Saag *et al.,* 1991; Schnittman *et al.,* 1990). Last, it is interesting to note that the significance of this signature might be restricted to the *vif* gene of B clade HIV-1. Indeed, a previous report of Wieland *et al.* (1997), identifying the sequences of *vif* genes of A and D clades of HIV-1 individuals of Uganda, did not point out a particular association between virus load and this Ser signature in Vif present in a large majority of A subtype isolates. However, in this study, the most relevant viral loads from individuals infected by subtype A HIV-1 were not measured, and therefore no clearcut conclusion might be possible.

The *in vivo* importance of Vif position 132 on the viral load parameter was confirmed *in vitro*. A reconstructed provirus encoding Vif with S in position 132 was less replicative in PBMC (restrictive cells for the *vif*- viruses) while the replication in SupT1 (permissive cells) was unaltered. A 5-fold reduction of virus replication in PBMC confirmed by a 10-fold decrease in infectivity of viruses normalized for the reverse transcriptase activity is in agreement with the mean decrease in viral loads within LTNP containing S or R at position 132 (4-fold decrease in copies per milliliter in plasma, see Table 3). It should have been important to control for the fact that primary isolates containing S132 replicate better than R132 viruses but most of the S132 containing viruses were not successfully isolated. Similarly, tentative cloning of complete primary *vif* genes did not allow us to go further in this approach as the *vif* genes from individuals with low viral load were not easily amplified and, the obtained sequences being genetically heterogeneous, a high number of clones for each individual would be necessary to compile conclusive data.

The functional role of position 132 of Vif is not known. However, it is interesting to note that residue 132 is just upstream of the second cysteine 133, which is essential for the infectivity of HIV-1 (Ma *et al.,* 1994). Since it has been shown that both Vif cysteine residues are not linked by an intramolecular disulfide bond (Sova *et al.,* 1997), the residue at position 132 may contribute to a local domain of Vif around cysteine 133 rather than to a

change in the overall conformation of the Vif protein. Although this residue at position 132 may be important for the optimal activity of Vif, the change in S to R at position 132 may be compensated by changes at other positions within Vif or by changes within other viral proteins. This compensatory effect may explain that the correlation which is observed *in vivo* between the signature at position 132 and the viral load is statistically significant, but that exceptions to this association do occur. Despite these restrictions, it is interesting to note that among the nine LTNP patients studied by Zhang *et al.* (1997b), six have the Ser residue and one has the homologous Thr residue at position 132.

Moreover, in a longitudinal study of mother-to-child transmission of HIV-1, Yedavalli *et al.* (1998) have characterized the *vif* sequences of six pairs of infant and mother and, interestingly, both pairs with asymptomatic mother and infant had a large majority of genotypes which code for Vif proteins with the S residue at position 132. These genotypes with S in position 132 are present in the mother and in the infant after perinatal transmission. It appears that there is no selective pressure to revert S into R, which, however, should easily occur since S and R codons differ by a single third base. Therefore, viruses with S132 are efficiently transmitted and well adapted to their host and the appearance of S at position 132 might rather occur at random. Once obtained, this residue is well conserved and may render the virus more adapted to immunocompetent individuals, moderately reducing its production.

Another mechanism related to the viral interference may be possible. Indeed the S variant may interfere with the R variant and therefore limits the outgrowth of the rapid variant. This interference mechanism has already been observed with a particular *vif* gene isolated from a patient infected by HIV-1 which inhibits *in trans* HIV-1 wild-type viruses in either permissive or nonpermissive cells (D'Aloja *et al.,* 1998). Interestingly, this variant *vif* gene contains several contiguous changes just upstream of the second conserved C residue at position 133, including the residue S132. Coinfection experiments with both variants containing S or R at position 132 and analysis of the resulting competition of both variants may allow this second mechanism to be assessed.

In this study, we have revealed that the Vif protein mutations may be subtly involved in the adaptation of viral replication within immunocompetent LTNP. This genetic determinant must be added to others, which were previously described as being involved in governing the rate of disease progression of HIV-1-infected patients. Among them, the polymorphism of the CCR5 second receptor is a good example showing that the relatively frequent heterozygous profile (+/Δ32) *CCR5* among infected individuals from western Europe is a factor influencing the rate of disease progression (Balfe *et al.,* 1998; Dean *et al.,* 1996; Huang *et al.,* 1996; Michael *et al.,* 1997). In our cohort, no direct correlation was observed between (+/Δ32) *CCR5* heterozygosity and viral load parameters. This result is in agreement with a previous study of Cohen *et al.* (1997), which found that *CCR5* heterozygosity is not the sole determinant for the immunologic and virologic phenotype of LTNP. Nevertheless, when combined genotypes of CCR5, CCR2, SDF1, and HLA genes were integrated in a statistical regression model, LTNP individuals could be significantly distinguished from progressors (Magierowska *et al.,* 1999). The recent discovery of a positive link between the CX3CR1 I249 M280 homozygosity and the more rapid progression to AIDS is another example of genetic risk factor in HIV/AIDS, confirming that the rate of disease appearance is dependent on multiple factors (Faure *et al.,* 2000).

It is noteworthy that a correlation was observed between an E to D change at position 25 of the V3 loop of the envelope glycoprotein and the viral load, with E associated with low viral load and D with high viral load (Candotti *et al.,* unpublished data). This last signature in V3 was previously observed in another study of 44 individuals classified as nonprogressors and slow progressors, with the D residue at position 25 of V3 associated with the increased rate of disease progression (Balfe *et al.,* 1998).

Grossly defective *nef* gene sequences have also been associated with LTNP status (Deacon *et al.,* 1995; Kirchhoff *et al.,* 1995; Salvi *et al.,* 1998). In our study, preliminary data on *nef* sequences reveal a high frequency of full-length *nef,* excluding the possibility that the deletions within *nef* contribute strongly to the LTNP status of the individuals included in our cohort. However, particular amino acid changes within Nef proteins affecting their functions are not excluded from involvement in the outcome of HIV-1 infection. Interestingly, a recent report by Kirchhoff *et al.* (1999) described that a large majority of *nef* sequences from individuals infected by HIV-1 are full-length and that a link was determined between specific amino acid changes within Nef and the progression status of the infected individuals. Further investigations will be necessary to gain a more precise understanding of the different parameters involved in LTNP status and in particular those involved in the subtle genetic changes of the auxiliary genes.

MATERIALS AND METHODS

Patients

Long-term nonprogressors were selected on the basis of their asymptomatic status, stable CD4 cell counts, and no anti-retroviral therapy (Candotti *et al.,* 1999). They were infected for at least 8 years and their CD4 cell counts were stable during the past 3 years preceding inclusion into the cohort. Late progressor controls were infected for at least 8 years without any symptoms and

not treated at the time of inclusion into the cohort but their CD4 cell counts had decreased at least 25% in the past 3 years. When viral load data were obtained following inclusion, individuals presenting viral loads greater than 50,000 copies/ml were rapidly excluded from the cohort, according to the rules of the French Ministry of Health applied at this period (beginning in 1995).

Isolation of DNA from PBMC

Peripheral blood mononuclear cells were isolated from heparinized blood using density-gradient centrifugation on Ficoll–Hypaque (Pharmacia, Uppsala, Sweden). DNA was isolated from approximately 10^6 frozen pelleted PBMC (Candotti *et al.,* 1999) according to the Boehringer Mannheim kit protocol (High Pure PCR template preparation kit). Less than 1 μg of DNA from PBMC was used for PCR amplification.

Cells

HeLa and SupT1 or C8166 cells were grown and maintained in Dulbecco's modified Eagle's medium and RPMI 1640 medium, respectively, supplemented with 10% fetal calf serum (FCS), antibiotics (penicillin and streptomycin), and 2 mM glutamine. PBMC were obtained from normal donor lymphopheresis samples which were aliquoted and stored in liquid nitrogen. After being thawed, PBMC were grown in RPMI supplemented with 10% FCS and phytohemagglutinin (PHA) for 3 days. Nonadherent cells containing most of peripheral blood lymphocytes were grown in RPMI supplemented by 10% FCS and 200 U/ml of interleukin-2 (IL2).

Transfections

Transfections were performed by the Fugen method as recommended by the manufacturer (Roche Inc., Paris). Briefly, HeLa cells were plated at 3.7×10^6 cells per 75-cm^2 flask and grown overnight. Cells were incubated with 32 μl of Fugen and 10 μg of appropriate plasmid DNA for 48 h. Virus was quantified in cell-free supernatants by measuring reverse transcriptase (RT) activity as previously described (Rey *et al.,* 1984).

Infection and virus propagation

Virus stocks were obtained 48 h after transfection of HeLa cells with appropriate viral DNA or from acutely infected SupT1 cells. SupT1 cells were infected by incubating 2×10^6 cells with 1 ml of virus supernatant at 37°C by gentle shaking. After centrifugation at 800*g* for 5 min, cells were resuspended in culture medium at the concentration of 5×10^5 cells/ml. Virus replication was assayed twice a week by determining the RT activity in the cell-free supernatant. SupT1 virus stocks were further titrated on C8166 to determine the infectious titer expressed as $TCID_{50}$ per milliliter and were used in different dilutions to infect PBMC. On day 3 after PHA stimulation, PBMC were infected with an equal m.o.i. of wild-type or mutant viruses. HIV-1 productions in cell cultures infected with serial fivefold dilutions of virus stocks were assayed in triplicate.

Isolation of RNA from plasma virus and cDNA synthesis

RNA from viral particles contained in 0.5 ml of plasma was extracted with RNAXEL II kit following the manufacturer's recommendation (Eurobio). Ethanol-precipitated RNA was resuspended in diethyl-pyrocarbonate-treated water and the cDNA synthesis was performed in 25 μl of reverse transcriptase buffer (Promega) containing 100 mM DTT, 0.2 μM of antisense primer E2 used for PCR, 40 μM dATP, dCTP, dGTP, and TTP, 10 U of RT (AMV RT Promega), and 20 U of RNasine (Promega) for 30 min at 40°C.

PCR amplification

A two-step PCR amplification, first with outer primers E1 (5′-CAAAATTTTCGGGTTTATTACAGGGAC, nt 4881 to 4907, sense) and E2 (5′-CTGCTATGTTGACACCCAATTCTG, nt 5788 to 5765, antisense) and then with inner nested primers I1M13 (5′-*TGTAAAACGGCCAGT*GGAAAGGTGAAGGGGCAGTAG, nt 4948 to 4968, sense) and I2M13 (5′-*CAGGAAACAGCTATGACC*TCTTAAGCTCCTCTAAAAGCTCTAGTG, nt 5631 to 5605, antisense), was performed to obtain the *vif* gene sequence. The nucleotides are numbered as in pNDK (Bouyac *et al.,* 1997; Spire *et al.,* 1989) and the M13 sequences are indicated in italics. PCR was performed with a 100-μl reaction mixture containing PCR buffer (Stratagene), 40 μM dATP, dCTP, dGTP, and dTTP, 0.2 to 1 μM of each outer primer pair, and 2.5 U of *Pfu* DNA polymerase. Twofold end-point dilutions of DNA extracted from PBMC or synthesized by *in vitro* RT reaction were used. The reaction was carried out at 94°C for 30 s, 60°C for 1 min, and 72°C for 1 min for 30 cycles. Five microliters of this PCR product amplification was used for a nested PCR according to the same protocol. The samples which were further analyzed originated from the highest twofold end-point dilutions which were positively amplified.

The amplified DNA products were purified by electrophoresis on a 1.5% low-melting agarose gel and extracted according to the DNA extraction kit protocol (QIAEX II). The *vif* gene nucleotide sequences were determined from both strands of amplified DNA with M13 primers by Genome Express (Grenoble, France).

vif gene cloning in recombined proviruses

(i) Construction of pNDKSm^R with Unique SacII and SalI Sites. In order to clone the *vif* genes from different viruses, pNDK plasmid (Spire *et al.,* 1989; Bouyac *et al.,* 1997) carrying NDK provirus was modified by the addi-

tion of two unique sites, *Sac*II and *Sal*I, bordering the *vif* gene of NDK. A two-step recombinant PCR methodology with the following *Sac*II and *Sal*I primers, VIFK7SacII (5′-GGTAGTA*CCGCGG*AGAAAAGTAAAGATCATTAGGG, nt 4994 to 5028, sense) and VIFK7SalI (5′-CACTCTTAAG-*GTCGAC*TAAAAGCTCTAATGTCC, nt 5634 to 5602, antisense), was used as previously described (Bouyac *et al.,* 1997). The nucleotide numbers are those of pNDK (Bouyac *et al.,* 1997; Spire *et al.,* 1989), the *Sac*II and *Sal*I sites are indicated in italics and the mutated nucleotides are underlined. The pNDKSacIISalI plasmid keeps the open reading frames surrounding *vif* of HIV-1 intact. The changes of the three nucleotides within the inserted *Sal*I and *Sac*II sites have no amino acid change in the IN domain of Pol and only two amino acid changes in Vpr, E to V at position 24 and E to D at position 25. Then, pNDKSm^R was constructed to eliminate the parental *vif* gene from pNDK. This construction was obtained by replacing the *vif* gene of NDK between the *Sac*II and *Sal*I sites of pNDKSacIISalI by a DNA coding for the resistance to streptomycine, Sm^R (Golden and Wiest, 1988).

(ii) YU2 vif Gene Cloning. Following the amplification of YU2 DNA by the outer primers E1 and E2 (see above), first-step PCR products were amplified by the VIFK7SacII and VIFK7SalI primers surrounding the *vif* gene of NDK and highly conserved in YU2 provirus. This second PCR product, which was naturally blunt-ended with *Pfu* DNA polymerase, was cloned into the pCR-Blunt vector (Zero Blunt PCR cloning kit, Invitrogen). After selection and propagation of appropriate plasmids in Top10 or XL1 Blue *Escherichia coli* bacteria, recombinant DNA was purified with the QIAEX II DNA extraction kit from Qiagen. After *Sal*I and *Sac*II (Biolabs) digestion, the YU2 *vif* gene was extracted on a low-melting agarose gel and cloned into the plasmid pNDKSm^R.

The same strategy was used for the YU2 *vif* gene mutant R132S. This mutant was obtained by two-step PCR mutagenesis on the YU2 *vif* gene with the primers VIFK7SacII and YU132B (5′-GCTTGATATTCACAACTAG-GACTAACTC, nt 5448 to 5421, antisense) and with the primers YU132A (5′-GAGTTAGTCCTAGTTGTGAATAT-CAAAGC, nt 5421 to 5448, sense) and VIFK7SalI. The YU-2 nucleotide coordinates were those previously described (Li *et al.,* 1992) and the mutated G to T sense nucleotide is underlined in the mutagenesis primers. The YU-2 *vif* gene with the mutation T159I has been generated with the following mutagenesis primers: YU159$^-$ (5′-CAAAGGTGGCTTTATCTTTTTTGGTG, nt 5527 to 5502, antisense) and YU159$^+$ (5′-CACCAAAAAAGATA-AAGCCACCTTTG, nt 5502 to 5527, sense). The reactions were carried out at 90°C for 30 s, 55°C for 45 s, and 72°C for 50 s for 30 cycles. The two PCR products were purified as previously described and mixed at an equimolar ratio for a second PCR with the primers VIFK7SacII and VIFK7SalI carried out at 90°C for 30 s, 60°C for 45 s, and 72°C for 1.5 min for 30 cycles.

Phylogenetic analysis of HIV-1 *vif* sequences and statistical testing

The nucleotide sequences of the *vif* genes (576 bp) from the 42 LTNP and the 8 late progressors were translated to corresponding amino acid sequences (192 amino acids). Zhang's sequences (Zhang *et al.,* 1997b) were obtained from the author and those of Sova *et al.* (1995) were obtained from GenBank. Alignments were performed with the ClustalW program (Thompson *et al.,* 1994). Phylogenetic trees of nucleotide and amino acid sequences were constructed by the neighbor joining method available in ClustalW (Thompson *et al.,* 1994).

Amino acids localized at certain positions and associated with specific groups of individuals were determined with VESPA, according to Korber and Myers (Korber and Myers, 1992). Statistical significance of the distribution of amino acid signatures among different groups of individuals was assessed by Student *t* test. Significant correlations ($P < 0.05$) between amino acid signatures and viral loads were assessed with Statview using the Mann–Whitney and Kruskall–Wallis tests.

Nucleotide sequence accession numbers

The major nucleotide sequences that we obtained for the *vif* genes from each patient have been submitted to GenBank under Accession Nos. AF143097 to AF143146.

APPENDIX

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Note added in proof. During the correction of this manuscript, Alexander *et al.* (L. Alexander, E. Weiskopf, T. C. Greenough, N. C. Gaddis,

M. R. Auerbach, M. H. Malim, S. J. O'Brien, B. Walker, J. L. Sullivan, and R. C. Desrosiers, *J. Virol.* **74,** 4361–4376, 2000) reported the *vif* sequences of eight LTNP and six of them have a residue other than Arg in position 132 including four individuals with Ser.

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